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Gene targeting in plants

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Although the generation of transgenic plants is now routine, the integration of foreign genetic information has so far been at random sites in the genome. We now present evidence for directed integration into a predicted location in the host plant genome. Protoplasts of transgenic tobacco (*Nicotiana tabacum*) plants carrying copies of a partial, non-functional drug-resistance gene in the nuclear DNA were used as recipients for DNA molecules containing the missing part of the gene. Molecular and genetic data confirm the integration of the foreign DNA through homologous recombination within overlapping parts of the protein coding region, resulting in the formation of an active gene in the host chromosome. This approach is referred to as gene targeting. The gene targeting frequency (the number of drug-resistant clones resulting from gene correction compared to the number of resistant clones from parallel experiments with a similar non-interrupted hybrid gene) was $0.5-4.2 \times 10^{-4}$. These experiments demonstrate the possibility of producing transgenic plants with desired modifications to a specific nuclear gene.

Key words: gene targeting/homologous DNA recombination/protoplast/transformation/transgenic tobacco plants

Introduction

There have been important advances in the past few years in gene transfer techniques with plants. The most common technique uses *Agrobacterium tumefaciens* as a vector (Rogers *et al.*, 1986). In addition, direct gene transfer to protoplasts (Paszkowski *et al.*, 1984; Hain *et al.*, 1985; Shillito *et al.*, 1985; Negrutiu *et al.*, 1987), protoplast fusion with liposomes containing DNA (Deshayes *et al.*, 1985), intranuclear microinjection of protoplasts (Crossway *et al.*, 1986; Reich *et al.*, 1986) and macroinjection into meristematic inflorescences (De la Pena *et al.*, 1987) are available.

Whatever the method, incoming DNA integrates efficiently into the nuclear genome but at random locations (Potrykus *et al.*, 1985; Ambros *et al.*, 1986; Nagy *et al.*, 1986; Wallroth *et al.*, 1986). It would be useful to be able to modify plant genes *in situ* at their natural position in the genome or to deliver foreign DNA into a predicted genomic location.

Although integration of transforming DNA into the homologous chromosomal DNA occurs efficiently in yeast, other fungi and *Dictyostelium discoideum* (Hinnen *et al.*, 1978; De Lozanne *et al.*, 1987; Miller *et al.*, 1987), non-homologous ('illegitimate') integration of foreign DNA into the genome of higher eukaryotic cells makes it difficult to assay the frequency of gene targeting to a desired locus. Only recently has the application of selection (Lin *et al.*, 1985; Thomas *et al.*, 1986; Doetschman *et al.*, 1987; Song *et al.*, 1987; Thomas and Capecchi, 1987) and screening (Smithies *et al.*, 1985) systems allowed comparison of the frequencies of homologous and illegitimate integration of transformed DNA in cultured mammalian cells. In plants the high transformation frequencies reported for DNA molecules with no homology to the plant genome (Shillito *et al.*, 1985; Negrutiu *et al.*, 1987) indicate efficient illegitimate integration. Therefore, the detection of gene targeting through homologous DNA recombination requires the utilization of a rigorous selection system such as selection for kanamycin resistance conferred by a gene coding for neomycin phosphotransferase [APH(3')II] which has provided tight selection both at the cellular and the plant level (Herrera-Estrella *et al.*, 1983; Paszkowski *et al.*, 1984).

We have constructed five strains of transgenic tobacco plants containing chromosomal copies of a non-functional, partially deleted, hybrid APH(3')II gene. Protoplasts of these plants were used for DNA mediated direct transformation with the missing part of the gene in order to correct its genomic counterpart. Here we report results of these experiments resulting in the desired modification of the genomic target gene sequence.

Results

Experimental design and the construction of the target plant strains

The bacterial APH(3')II gene under the control of plant viral expression signals (Figure 1A and B) was used in the construction of two sets of complementing plasmid molecules which could form an active gene through homologous recombination (Figure 1A). One set (*3' deletions*) contained the promoter region and 5' part of the structural gene (Figure 1A;b,f). The other set (*5' deletions*) contained the 3' end of the APH(3')II structural gene and the signals for polyadenylation (Figure 1A;c,d,g). With combinations of these two types of plasmids we were able to choose defined lengths of homology in the central part of the APH(3')II gene. Details of the plasmid constructions are described in the legend to Figure 1.

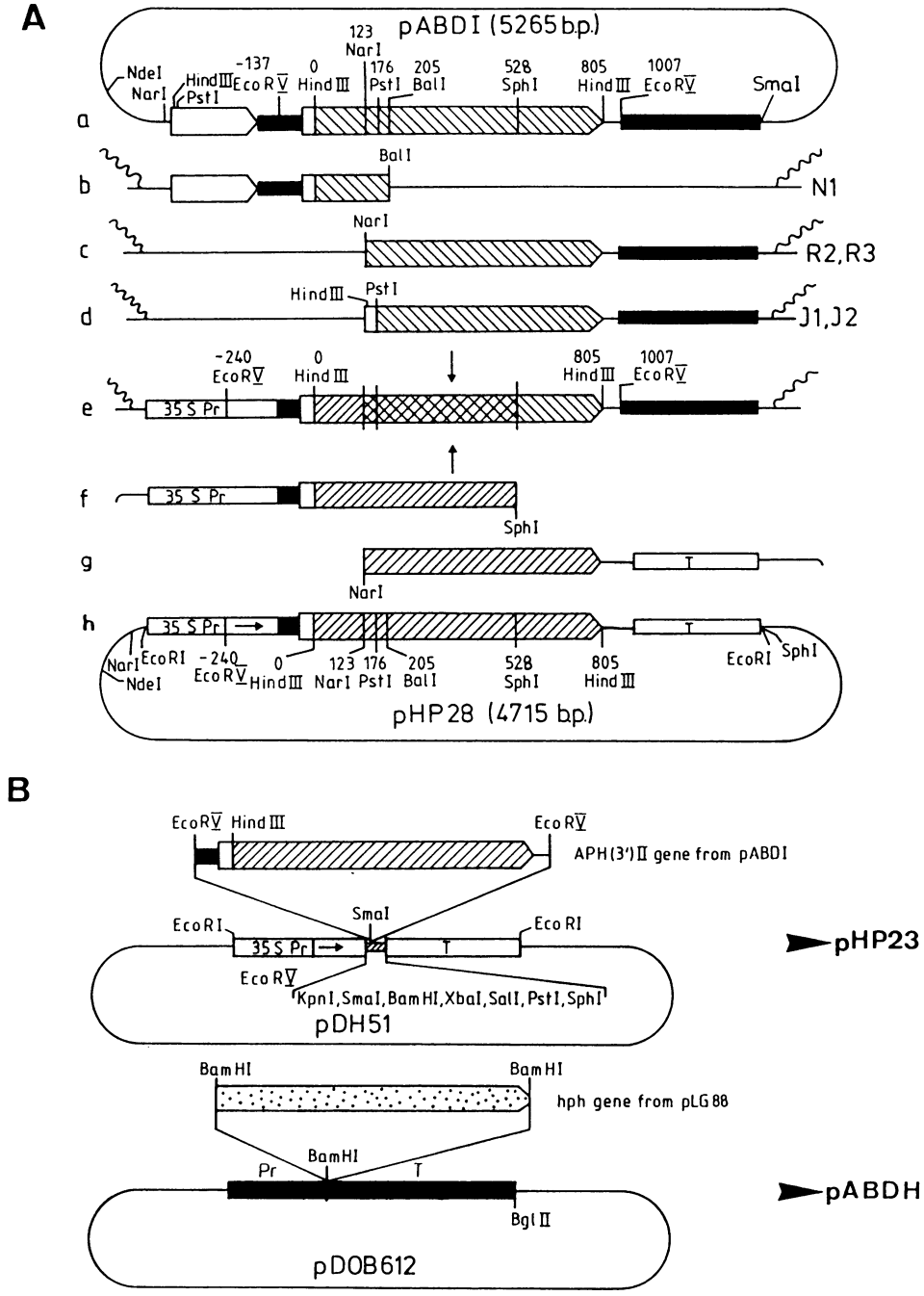
A series of transgenic tobacco lines were produced as recipients for gene targeting by introducing the 5' or 3' deletion derivatives of the APH(3')II gene (Figure 1A;b,c,d) into mesophyll protoplasts using co-transformation (Schocher *et al.*, 1986) with a selectable gene for hygromycin resistance (Figure 1B). Seven random hygromycin-resistant clones for

each deletion derivative were screened by Southern blot hybridization. Five clones were chosen (N1, R2, R3, J1, J2) containing the expected gene structure for particular APH(3')II deletion derivatives of plasmid pABDI (Figure 1A;b,c,d). The copy numbers of the truncated APH(3')II gene (intact deletion derivatives) in the cell lines J1, J2, N1, R2 and R3 were estimated by Southern blot reconstruction to be ~3, 7, 5, 2 and 10, respectively. Previous experiments with direct gene transfer into tobacco protoplasts have shown that although high copy numbers of integrated DNA are often found, in 77% of cases the integrated DNA segregates as one locus (Potrykus *et al.*, 1987). Thus the copy numbers given above probably represent DNA integrated in one locus in the plant genome and these copies can be often found as concatamers (Czernilofsky *et al.*, 1986).

Transgenic plants were regenerated and the integration of the constructs into the nuclear genome was verified by their mitotic stability in culture and transmission through male gametes (tested by Southern blot analysis on progeny from crosses to wild-type). R_0 hemizygous plants were propagated as shoot cultures as a source of protoplasts.

Gene targeting

All five lines were used in targeting experiments (Table I). Plasmid DNA containing complementing parts of the APH(3')II hybrid gene was introduced by direct gene transfer into protoplasts of the corresponding plant strains as follows: plasmid pHP28ΔNarI (Figure 1A;g) into strain N1 (Figure 1A;b), plasmid pHP28ΔSphI (Figure 1A;f) into strains R2, R3, J1 and J2 (Figure 1A;c,d). From three independent experiments involving a total of 1.68×10^8 protoplasts (Table I), eight kanamycin-resistant cell clones were obtained



from recipient strains J2 and R3. A positive APH(3')II activity test of the clones (data not shown) confirmed the nature of this resistance in each case. No kanamycin-resistant clones were observed in corresponding control experiments where wild-type SR1 protoplasts were used or where non-complementary deletion derivatives were supplied to protoplasts of the five lines (a total of 1.1×10^8 protoplasts).

The frequency of the appearance of kanamycin-resistant clones per treated protoplast was compared to the frequency of transformation with a similar, non-interrupted hybrid gene (plasmid pHP28) in parallel treatments. The ratio (the relative targeting frequency, RTF) varied from 5.4×10^{-5} for line R3 to $0.83-4.2 \times 10^{-4}$ (reproducible gene targeting) in line J2 (Table I). The expression signals of the gene predicted to result from targeting are identical to those of the intact reference gene. The only difference between these two genes is that the poly(A) addition site in the reference gene is 280 bp closer to the coding sequence.

Molecular analysis of the targeting events

The 3' and 5' APH(3')II gene deletions were derived from two hybrid genes with differing restriction sites in the promoter and 3' gene regions. Homologous recombination between the deletions should result in a marker gene with a new combination of flanking segments, distinguishable from the two constructs previously existing (pABDI and pHP28). Southern analysis of four J2 complemented clones revealed a predicted *Eco*RV fragment of the new marker gene (Figure 2A; lanes 3–6). This confirms recombination in the central region of homology of the two deletion derivatives, and rules out the possibility of transformation with contaminating parental plasmids. A reprobing of the filters with probes specific for 5' part and 3' part of the gene [for 5' fragment *Eco*RV(−240)−*Pst*I(176) and *Hin*dIII(0)−*Pst*I(176) and for 3' *Pst*I(176)−*Hin*dIII(805), Figure 1] revealed that both probes hybridized with the same *Eco*RV fragment as the complete probe. Southern analysis also showed that not all copies of the integrated APH(3')II deletions were corrected: (as seen in Figure 2B, some 634 bp *Hin*dIII fragments were not converted to new 805 bp fragments).

Analysis of progeny plants of J2-7

The ability to regenerate fertile plants from cell clones provides a unique opportunity to verify the molecular data of gene targeting by genetic crosses. Genetic analysis of

Table I. Efficiency of gene targeting

Plant strain	Number of protoplasts treated × 10^6			Number of kanamycin^r clones with pHP28			Number of kanamycin^r clones with complementing deletion			Relative targeting frequency		
	Experiment no.			Experiment no.			Experiment no.			Experiment no.		
	1	2	3	1	2	3	1	2	3	1	2	3
N1		18	8.4		12960	6888		0	0			
R2	12	18	8.4	6950	21660	8470	0	0	0			
R3	12	18	8.4	7110	18480	6440	0	1	0		5.4×10^{-5}	
J1		18	8.4		6450	5264		0	0			
J2	12	18	8.4	11920	11940	6020	5	1	1	4.2×10^{-4}	8.4×10^{-5}	1.7×10^{-4}

The efficiency of gene targeting was calculated as the ratio of the number of kanamycin^r clones resulting from gene targeting and the number of kanamycin^r clones obtained by transformation with an intact APH(3')II gene (as estimated from the number of transformants obtained by transformation of 2.4×10^6 protoplasts in two independent treatments).

Fig. 1. (**A**) Scheme of the gene targeting experiments. Two plasmids (lines a and h) carrying different constructs of the APH(3')II selectable hybrid marker gene were used for the construction of complementing deletion derivatives. **Line a**: plasmid pABDI which has been previously described (Paszkowski *et al.*, 1984). **Line b**: pABDIΔBalI—deletion derivative of plasmid pABDI obtained by deletion of a *Bal*I−*Sma*I fragment of pABDI. DNA of this plasmid was linearized at the *Nde*I site and integrated into the tobacco nuclear genome, by co-transformation with pABDH plasmid (B), generating the N1 strain of target tobacco. **Line c**: pABDIΔNarI—deletion derivative, obtained by deletion of the *Nar*I fragment of plasmid pABDI. pABDIΔNarI was introduced into the tobacco genome by co-transformation, generating the R2 and R3 strains. **Line d**: pABDIΔPstI—deletion derivative of plasmid pABDI constructed by the deletion of the *Pst*I fragment of pABDI, which after introduction into the tobacco genome created target strains J1 and J2. **Line e**: map of the predicted hybrid gene formed as a product of gene correction by homologous recombination of deletion derivatives. **Line f**: pHP28ΔSphI—deletion derivative of plasmid pHP28 obtained by deletion of its *Sph*I fragment. **Line g**: pHP28ΔNarI—deletion derivative of pHP28 obtained by deleting its *Nar*I fragment. **Line h**: plasmid pHP28 [construction of which is shown in (B)]. Numbers above the restriction sites in lines a and h indicate their relative positive (in bp) to the *Hin*dIII '0' at the beginning of the APH(3')II protein-coding region. In lines b, c, d, e, f and g only a few crucial restriction sites are displayed but the pattern of drawing consistent in all lines indicates the same DNA stretches with corresponding restriction sites. A single line represents DNA from the pUC type plasmids (Yanisch-Perron *et al.*, 1985); the solid box represents promoter and terminator regions of the 19S RNA transcript of Cauliflower Mosaic Virus (CaMV); the broad open box represents the end of open reading frame 5 (ORF5) and the beginning of the ORF6 of CaMV; narrow open boxes represent promoter (Pr) and terminator (T) regions of the 35S RNA transcript of CaMV; the box with diagonal lines from left to right−top to bottom and the box with diagonal lines from right to left−top to bottom represent APH(3')II protein-coding regions from plasmids pABDI and pHP28 respectively, and the cross-hatched box in line e represents a region of homology which should permit homologous recombination to restore gene function during targeting; wavy lines represent nuclear DNA. (**B**) Construction scheme of the plasmids pHP23, pHP28 and pABDH. Plasmid pHP23 was constructed by the insertion of an *Eco*RV fragment of plasmid pADBI (Paszkowski *et al.*, 1984) (A) containing the APH(3')II structural gene and part of the 19S RNA promoter sequence of CaMV into the *Sma*I site of the plasmid pDH51 (Pietrzak *et al.*, 1986) which contains the CaMV 35S RNA promoter and terminator regions separated by a synthetic poly-linker (narrow box with diagonal lines). Plasmid pHP23 was modified, giving rise to pHP28, by deleting part of the polycloning linker between the *Bam*HI and *Sph*I restriction sites and recloning of the *Eco*RI fragment containing the hybrid gene into plasmid pUC19 in order to facilitate creation of further deletion derivatives. Plasmid pABDH was constructed by the insertion of the *Bam*HI fragment of plasmid pLG88 (Gritz and Davies, 1983) containing the protein-coding region of hygromycin phosphotransferase (hph) into the *Bam*HI site of pDOB612 (Balazs *et al.*, 1985). The dotted box represents the protein-coding region of the *hph* gene; the remaining symbols are as in (A).

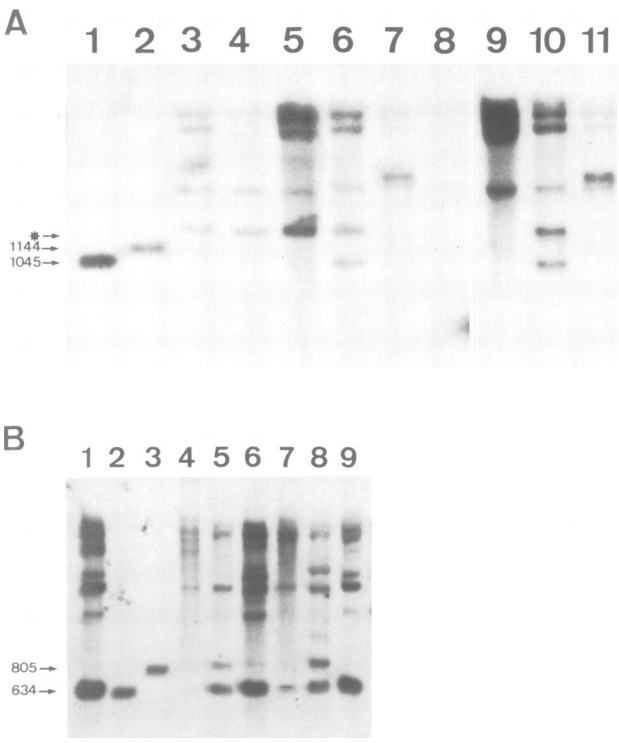


Fig. 2. Southern blot analysis of genomic DNA from kanamycin-resistant clones obtained by gene targeting. DNA was hybridized with a radioactively labeled *Hin*dIII fragment of the pABDI spanning the protein-coding region of the APH(3′)II gene. (**A**) DNA cleaved with *Eco*RV. **Lane 1**: size marker of 1045 bp (six copies reconstruction). **Lane 2**: size marker of 1144 bp (*Eco*RV fragment of pABDI, three copies reconstruction). Lanes 3–6: genomic DNAs of four kanamycin^r clones of line J2 transformed with complementing parts of the APH(3′)II hybrid gene (plasmid pHP28ΔSphI, Figure 1A;f). **lane 3**: genomic DNA of plant J2-1. **Lane 4**: genomic DNA of plant J2-2. **Lane 5**: genomic DNA of plant J2-7. **Lane 6**: genomic DNA of plant J2-5. **Lane 7**: genomic DNA of strain J2 transformed with plasmid pHP28. **Lane 8**: genomic DNA of strain J2. Lanes 9–11: different Southern blot analyses. **Lane 9**: DNA of plant J2 (compare *lane 8*). **Lane 10**: DNA of plant J2-5 (compare *lane 6*). **Lane 11**: DNA of strain J2 transformed with plasmid pHP28 (compare *lane 7*). (**B**) DNA cleaved with *Hin*dIII. **Lanes 1** and **9**: genomic DNA of J2. **Lane 2**: plasmid pABDIΔPstI restricted with *Hin*dIII (two copies reconstruction). **Lane 3**: plasmid pABDI restricted with *Hin*dIII (two copies reconstruction). Lanes 4–8: genomic DNA of five kanamycin^r clones obtained by transformation of J2 with an APH(3′)II deletion derivative (plasmid pHP28ΔSphI, Figure 1;f). **Lane 4**: genomic DNA of plant J2-1. **Lane 5**: genomic DNA of plant J2-2. **Lane 6**: genomic DNA of plant J2-7. **Lane 7**: genomic DNA of plant J2-5. **Lane 8**: genomic DNA of plant J2-6. Sizes of the fragments (in bp) displayed next to the arrows. The expected newly formed *Eco*RV fragment of 1247 bp (see text) is marked by an '*'.

progeny from the R_0 J2 target plant showed that the deletion derivative used for target recombination was integrated together with rearranged DNA fragments bearing APH(3′)II sequences which formed a characteristic *Eco*RV or *Hin*dIII pattern of the target locus on a Southern blot (Figures 2 and 3). If this genomic locus accepted new DNA correcting the J2–pABDIΔPstI deletion derivative (Figure 1A;d) to an active hybrid APH(3′)II gene, then the new APH(3′)II gene should be genetically linked with this particular pre-existing restriction pattern. Out-crosses of the kanamycin-resistant targeted plant J2-7 (pollination with SR1 pollen) and Southern blot analysis of the kanamycin-resistant progeny showed co-segregation of the newly formed

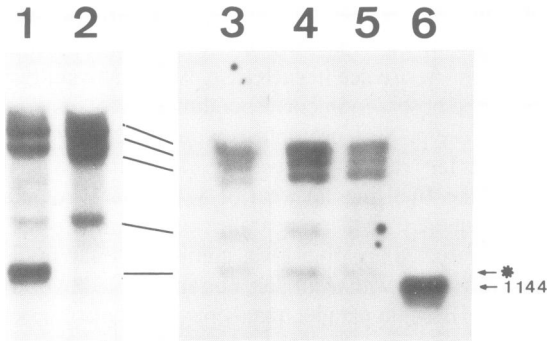


Fig. 3. Southern blot analysis of genomic DNA from the F1 progeny of a kanamycin-resistant plant. J2-7 DNA was cleaved with *Eco*RV endonuclease (compare with Figure 2A where J2-7 is displayed in lane 5). **Lane 1**: DNA of J2-7 plant. **Lane 2**: DNA of J2 plant. **Lanes 3–5**: DNA of three progeny plants of kanamycin^r J2-7 which was pollinated with wild-type SR1 pollen. **Lane 6**: size marker 1144 bp. The number next to the arrow indicates the length of the fragment in bp. '*'—marker points to the expected newly formed 1247 bp *Eco*RV fragment (compare with Figure 2A).

APH(3′)II gene and the locus specific restriction pattern (Figure 3). These results are consistent with the notion that correction of the APH(3′)II gene occurred at its original integration site on the plant genome, i.e. correction of the incoming plasmid sequence followed by integration elsewhere is unlikely (Song *et al.*, 1987).

Discussion

We have presented here a simple set of experiments designed in such a way that the results should clearly support or clearly refute the possibility of integration of the foreign DNA into an homologous location in the plant genome.

A 5′ deletion of the APH(3′)II gene integrated in a chromosome should, upon homologous recombination with an incoming 3′ deletion derivative of this gene, result in the restoration of gene function. Active APH(3′)II should confer kanamycin resistance to plant cells. This was indeed observed in three independent experiments yielding a total of eight kanamycin-resistant clones. Kanamycin-resistant clones were not observed in the corresponding control treatments indicating a clean, reliable selection system.

The restoration of gene function via gene targeting should be a consequence of the reconstruction of a predicted physical structure of a hybrid APH(3′)II gene in the genome. In order to facilitate analysis of this structure, we have marked the flanking sequences of the recombining part of the gene with restriction sites. Thus the gene reconstructed by gene targeting should acquire an expected structure which had never existed, in a cloned form, as part of a plasmid. This allowed exclusion of artefacts which might be caused by impurities in the transforming DNA and allowed the use of a 1247 bp *Eco*RV junction fragment, formed by targeting, as a molecular marker for the targeting event. Analysis of four independent kanamycin-resistant clones revealed the presence of this diagnostic fragment and also a new 805 bp *Hin*dIII fragment which is internal to the *Eco*RV fragment. That four independent kanamycin-resistant clones all contained the predicted restriction fragments indicates the reproducibility of a particular mechanism of gene correction. The full elucidation of this mechanism will need further

studies involving new plant strains with single copy targets. However, since the incoming DNA was in a linear form and we did not detect double-deleted gene expected from a single reciprocal crossover event (after possible plasmid circularization), gene correction was probably by double reciprocal recombination or gene conversion, as described for animal cells (Thomas *et al.*, 1986; Song *et al.*, 1987; Thomas and Capecchi, 1987). The complementing deletion derivatives were constructed on the same type of bacterial plasmid (Figure 1) and some homologous DNA sequences outside the marker gene flanking the deletion derivatives could also be involved in the recombination or gene conversion processes.

Southern hybridization has shown the reconstruction of a *Hin*dIII fragment (805 bp) covering the protein-coding region of APH(3′)II gene (Figure 2B), suggesting that a very precise correction of the hybrid gene occurred in most of the cases. This is to be expected because of the tight, direct selection based on the reconstruction of the reading frame of the gene. In some cases, however, the relative intensity of the bands on the Southern blots is puzzling and not easy to explain. For example *Eco*RV and *Hin*dIII DNA analysis of plant J2-7 [Figure 1A (lane 5) and B (lane 6)] showed that the strong 1247 bp *Eco*RV band has reproducibly resulted in only a relatively weak internal 805 bp *Hin*dIII band. This can only be explained by the differential accessibility of particular restriction sites for enzymatic digestion or, alternatively, it is possible that the recombination process might result in the accumulation of silent mutations which would not alter the APH(3′)II gene expression but cause a change in a restriction enzyme recognition sequence. This could be reflected in the change of relative intensity of *Hin*dIII and *Eco*RV bands on Southern blots. The precision of the targeting process can only be fully defined by recloning and sequencing of the rebuilt genes.

The genetic data indicating 'locus-linked' sexual transmission of the reconstructed gene are consistent with the suggestion that the gene correction occurred at the genomic location of the target sequence. However a more extensive genetic analysis is needed to confirm this suggestion. It is however interesting that, as compared to the J2-7 plant, a lower relative intensity of 1247 bp *Eco*RV band was observed in the progeny (Figure 3). This might be an indication of meiotic instability of some of the newly formed APH(3′)II gene copies. Such instability of genome integrated foreign DNA was observed before (Potrykus *et al.*, 1985).

The lack of correlation between the copy number of the integrated incomplete gene and the RTFs, observed in mammalian cells, indicated that the initial homology recognition step was not limiting (Thomas *et al.*, 1986). We also observed no obvious correlation; however, successful targeting was obtained only in lines containing the highest copy number of the target sequence (7 copies for J2 and 10 copies for R3). The absence of correction in some strains harboring deletion derivatives might suggest that the homology region was too short (82 bp for strain N1) or that the co-transformed APH(3′)II deletion derivatives accumulate secondary mutations preventing their later correction; however, co-transformation of functional non-selectable genes to plants is normally very efficient (Schocher *et al.*, 1986). It is also possible that some genomic regions are more or less accessible for gene expression or homologous exchange with foreign DNA as has been suggested for animal cells (Lin *et al.*, 1985). It should be noted that the locus used as a target had been artificially created by transformation. This has to be considered when extrapolating the RTFs obtained here to an endogenous plant gene. It is very possible that RTFs might be different for every gene.

All corrected kanamycin-resistant clones were obtained with the complementing part of the gene in a linear form, linearized within the homologous region, and thus providing recombinogenic ends (see legend to Table I). In parallel experiments involving 2.4×10^7 J2 protoplasts, super-coiled plasmid DNA and plasmid DNA linearized outside of the APH(3′)II gene were ineffective. These observations already point to preferred structures for incoming targeting DNA but more investigation of this point is required.

The frequencies of gene targeting reported for mammalian systems varied depending on the cell material and DNA delivery method used (10^{-5} to 10^{-2}) (Lin *et al.*, 1985; Smithies *et al.*, 1985; Thomas *et al.*, 1986; Doetschman *et al.*, 1987; Song *et al.*, 1987; Thomas and Capecchi, 1987). The RTFs reported here for plants are low but are comparable to some mammalian cells. Experiments aiming for higher targeting frequency are in progress but the data presented here are a first, important step towards precise modification, correction and directed mutagenesis of the plant genome. This should prove useful in studies of the organization and function of plant genes, as well as in predictable genome alterations useful in plant breeding.

Materials and methods

Plasmid constructions

Methods used for plasmid constructions and the isolation of plasmid DNA were as described in Maniatis *et al.* (1982). Restriction endonucleases were obtained from Boehringer, Biolabs and BRL. T4 ligase and T4 polymerase were from Biolabs.

Construction of transgenic target tobacco lines

Transgenic target tobacco lines were produced by the co-transformation of the deletion plasmids (10 μg DNA) linearized with *Nde*I, mixed with 2 μg of *Nde*I linear pABDH DNA (Figure 1B) and 40 μg of carrier calf thymus DNA. Plasmid and carrier DNA was sterilized by ethanol reprecipitation as described in Paszkowski and Saul (1986) and introduced into tobacco (*Nicotiana tabacum* cv. Petit Havana line SR1, Maliga *et al.*, 1973) mesophyll protoplasts using the chemical transformation method developed by Negrutiu *et al.* (1987). Hygromycin selection, starting in the second week of culture, was performed in a bead-type culture system (Shillito *et al.*, 1983) at an antibiotic concentration of 12 mg/l for 6 weeks. After the selection period, resistant cell clones of 1−2 mm diameter were transferred to agar-solidified antibiotic free medium. Conditions for cell growth and plant regeneration were as described in detail by Saul *et al.* (1987).

Gene targeting

N.tabacum lines containing parts of the selectable marker gene integrated in the genome were transformed with the complementing gene deletions as described in the text. A total of 10 μg of the plasmid DNA harbouring the deletion derivative was linearized with restriction endonucleases as follows: plasmid pHP28ΔNarI (Figure 1A;g) with *Nar*I and plasmid pHP28ΔSphI (Figure 1;f) with *Sph*I. The method of transformation and selection of kanamycin-resistant clones (resistant to 50 mg/l kanamycin sulphate) was analogous to that used for the production of hygromycin-resistant clones (legend Figure 1A). Growth and regeneration media contained 50 mg/l kanamycin sulphate.

Southern blot analysis of plant DNA

Plant DNA was isolated using the method described in detail by Paszkowski and Saul (1986). Approximately 5 μg of genomic DNA was cleaved with *Eco*RV or *Hin*dIII and separated by electrophoresis in a standard 1% agarose

gel (Maniatis *et al.*, 1982). The radioactive probe was obtained by the random primer method (Feinberg and Vogelstein, 1983) (10^8 c.p.m./μg of DNA). Southern blot analysis was performed as described earlier (Paszkowski and Saul, 1986).

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